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Plasma homocysteine and *S*-adenosylmethionine in erythrocytes as determinants of carotid intima-media thickness: different effects in diabetic and non-diabetic individuals
The Hoorn Study

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Abstract

Objective: Hyperhomocysteinemia is a risk factor for atherothrombosis. Through unknown mechanisms, individuals with type 2 diabetes appear particularly susceptible. We determined whether components of homocysteine metabolism are associated with intima-media thickness in individuals with and without type 2 diabetes. *Methods and results:* In a cross-sectional design, we studied 231 Caucasian individuals, 60.6% having type 2 diabetes. We measured fasting homocysteine, vitamin B_6 and vitamin B_{12} in plasma, and folate, *S*-adenosylmethionine and *S*-adenosylhomocysteine in plasma and erythrocytes. A homocysteine concentration >12 μmol/l was associated with a greater intima-media thickness of $+0.07$ mm (95% CI, $+0.01$ to $+0.13$; $P=0.03$) among diabetic individuals and of -0.004 mm (95%CI, -0.08 to $+0.07$; $P=0.92$) among non-diabetic individuals. An erythrocyte *S*-adenosylmethionine concentration above >4000 nmol/l was associated with a smaller intima-media thickness of -0.04 mm (95%CI, -0.10 to $+0.02$; $P=0.17$) for diabetic individuals versus -0.12 mm (95%CI, -0.20 to -0.36; $P=0.005$) for non-diabetic individuals. *Conclusions:* With regard to carotid intima-media thickness, individuals with diabetes appear more susceptible to the detrimental effects of homocysteine than non-diabetic individuals. In addition, diabetic individuals may lack the protective effect on the vascular wall conferred by high concentrations of *S*-adenosylmethionine. These findings may help explain why hyperhomocysteinemia is an especially strong risk factor for atherothrombosis among individuals with type 2 diabetes.

Keywords: Homocysteine; *S*-adenosylmethionine; Intima-media thickness; Type 2 diabetes; Atherosclerosis

1. Introduction

An elevated plasma homocysteine concentration is associated with an increased risk of atherothrombosis, especially in individuals with type 2 diabetes [1–4]. It has not been fully elucidated by which mechanism(s) hyperhomocysteinemia exerts its adverse effects on the arterial wall. In fact, it is even unclear whether homocysteine itself is the causal factor. Homocysteine is produced during methionine metabolism via the adenosylated compounds *S*-adenosylmethionine and *S*-adenosylhomocysteine. *S*-adenosylmethionine is a methyl donor in numerous reactions [5]. Homocysteine can be remethylated to methionine in a reaction that requires

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folate and vitamin B_{12}. Alternatively, homocysteine can be transsulfurated to cysteine, which requires vitamin B_6 [5]. There is evidence that implicates one or several components of homocysteine metabolism in the process of atherosclerosis. Specifically, a low folate concentration (which is associated with hyperhomocysteinemia), a low *S*-adenosylmethionine concentration, a high *S*-adenosylhomocysteine concentration, and (or) a low ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine may be more directly related to vascular damage than homocysteine [6–10].

Individuals with type 2 diabetes have a 2- to 4-fold higher risk of cardiovascular disease compared with the non-diabetic population. The mechanisms that relate type 2 diabetes to cardiovascular disease are only partly understood. An increased susceptibility of individuals with type 2 diabetes to the detrimental effects of homocysteine has been demonstrated repeatedly [2–4]. However, as is the case for pro-atherosclerotic effects of homocysteine in general, the mechanisms explaining this increased vulnerability of patients with type 2 diabetes are unknown.

Carotid intima-media thickness is a marker of atherosclerosis and predicts cardiovascular events [11]. We set out to determine whether homocysteine and/or other components of homocysteine metabolism are independently associated with carotid intima-media thickness, and whether the associations differ between individuals with and without type 2 diabetes.

2. Methods

2.1. Participants

For the present cross-sectional investigation we used data from the 2000 follow-up examination of the Hoorn study and, to increase the number of individuals with type 2 diabetes, data from a recent diabetes screening study, both of which were population-based [12,13]. The Hoorn study is a population-based cohort study of glucose tolerance in the general population, which started in 1989 and consisted of 2484 participants [12]. In 2000, a follow-up examination was carried out among selected surviving participants, then aged 60–85 years old, who had agreed to be re-contacted for follow-up examinations. Participants were selected according to glucose metabolism status as the previous follow-up medical examination in 1996–1998.

We invited all those who had diabetes in 1996–1998 ($n = 176$). In addition, we invited random samples of subjects who had normal glucose tolerance ($n = 705$) or impaired glucose tolerance ($n = 193$). Of 1074 individuals thus invited, 648 (60%) participated. In addition, we invited 217 individuals with newly diagnosed type 2 diabetes who were diagnosed in a population screening study among the 50–75 years old population held in 2000, of whom 188 (87%) participated [13]. Thus, the number of participants was $n = 836$. For reasons of efficiency, we investigated in the present study a random sample ($n = 231$) of this population-based cohort with oversampling of participants with type 2 diabetes. Of these, 140 (60.6%) had type 2 diabetes, 22 (9.5%) had an impaired and 69 (29.9%) a normal glucose metabolism according to the WHO guidelines [14]. All participants gave informed consent for this study, which was approved by the local ethics committee.

2.2. Measurement of carotid intima-media thickness

A single observer (RMA H), who was unaware of participants' clinical or laboratory characteristics, measured intima-media thickness of the right common carotid artery by means of an ultrasound scanner equipped with an 7.5 MHz linear array probe (Pie 350 Series, Pie Medical BV, Maastricht, The Netherlands). The ultrasound scanner was connected to a personal computer equipped with vessel wall movement detection software and an acquisition system (Wall Track System 2, Pie Medical BV). This integrated set-up enables measurement of intima-media thickness, as described in detail elsewhere [15]. Briefly, after 15 min of supine rest, the right common carotid artery was visualized in B-mode. An M-line perpendicular to the artery was then placed at the site of measurement. After switching to M-mode, data acquisition was enabled after identification of the lumen of the artery (trackball-assisted) in a real-time A-mode presentation on the computer screen. Ultrasound data were then obtained during three consecutive measurements, each consisting of a 4-s period (including ca. four to seven heartbeats) triggered by the R-top of a simultaneously recorded electrocardiogram. The first radio-frequency signal was displayed on the screen, enabling the observer to check whether the markers, automatically positioned by the Wall Track System, coincide with the anterior (adventitia-media) and posterior (media-adventitia) vessel wall reflections in the diastolic phase of the cardiac cycle. The cumulative radio-frequency signals were then digitized and stored into the computer memory. Based on the radio-frequency signals of the common carotid artery posterior wall, the distance from the leading edge interface between lumen and intima to the leading edge interface between media and adventitia was calculated automatically as the intima-media thickness-complex. The mean intima-media thickness of the three consecutive measurements was used in the analyses. The common carotid artery was measured approximately 10 mm from the proximal beginning of the carotid bulb. The intra-observer inter-session coefficient of variation for intima-media thickness measurements at the start of the study ($n = 7$; 2-weeks' interval) was on average 11%.

2.2.1. Blood pressure measurements

Throughout the ultrasound examination, systolic and diastolic blood pressures were assessed in the left upper arm at 5 min intervals with an oscillometric device. (Collin Press-Mate, model BP-8800, Komaki-City, Japan). The mean of on average 16 measurements was used in the analyses.

2.3. Other measurements

Participants were invited to the study center after an overnight fasting period. We measured weight barefoot with light clothes only, height, and waist and hip circumferences. We calculated the body mass index (weight/height^2) and the waist-to-hip circumference ratio. Blood samples were drawn for the measurement of homocysteine and its precursors, B vitamins, lipids (total cholesterol, high-density-lipoprotein cholesterol, triglycerides), creatinine, insulin and hematocrit. We estimated creatinine clearance according to the Cockroft-Gault formula. Hypertension was defined according to the criteria of the Dutch Society of General Practitioners in use when these data were collected: systolic blood pressure ≥ 160 mmHg, diastolic blood pressure ≥ 95 mmHg, and/or the use of antihypertensive medication. Participants were classified as current smokers or nonsmokers. Information on prior cardiovascular diseases (myocardial infarction, angina pectoris, stroke and peripheral atherosclerotic disease) was obtained from the subjects by means of a translated version of the Rose questionnaire from the London School of Hygiene [16].

2.3.1. Sample preparation

All samples were processed within 30 min, stored at $-80\,^{\circ}C$ (except for lipids and creatinine, which were stored at $-20\,^{\circ}C$), and analyzed within 3 months. After collection, ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood samples were placed on ice for the determination of homocysteine, *S*-adenosylmethionine and *S*-adenosylhomocysteine. For *S*-adenosylmethionine and *S*-adenosylhomocysteine measurements, we deproteinized samples immediately by adding 0.625 ml of a 10% perchloric acid solution to 1 ml plasma and by adding 1 ml of 5% perchloric acid to 1 ml whole blood, followed by mixing [10]. We added 0.5 mg of ascorbic acid to 0.5 ml of serum for the determination of total folate [10]. For the determination of total folate in erythrocytes, 1 ml of reagent with ascorbic acid, human serum albumin and sodium azide (ACS:180, Chiron Diagnostics) was added to 50 μl of whole blood.

2.3.2. Determination of homocysteine, *S*-adenosylmethionine, *S*-adenosylhomocysteine and total folates

Total plasma homocysteine was determined with an automated fluorescence polarization immunoassay on an Abbott IMx analyzer (interassay CV, 4%) [17]. We used tandem mass spectrometry for the determination of *S*-adenosylmethionine and *S*-adenosylhomocysteine in plasma and whole blood, as previously described (intra-assay CV, 4% for both determinations; interassay CV, 8 and 6%, respectively) [18]. We measured total folate in red blood cell hemolysate and serum, and vitamin B_{12} in serum by means of automated chemiluminescence (Chiron Diagnostics ASC:180® Automated Chemiluminescence Systems). The intra-assay and interassay CVs for total folate were 4 and 5%, respectively, and 4 and 5% for vitamin B_{12}. Erythrocyte concentrations of the metabolites were calculated by multiplying the difference between plasma or serum and whole blood values by 100 per hematocrit. We used high-performance liquid chromatography to measure plasma concentrations of vitamin B_6, with an interassay CV of 7% [19].

2.3.3. Determination of lipids, creatinine and insulin

We determined serum total cholesterol, HDL-cholesterol and triglycerides by enzymatic techniques (Boehringer-Mannheim, Mannheim, Germany) and serum creatinine by means of modified Jaffé method. Insulin was determined using a two-site immuno radiometric test. Paired monoclonal antibodies were used (Medgenix, Diagnostics, Fleurus, Belgium).

2.4. Statistical analyses

All analyses were performed with SPSS 10.1 for Windows 95. Differences between groups in continuous variables were tested with Student's *t*-test; in case of a skewed distribution with the Mann–Whitney test; and in case of percentages with the χ^2-test. Adjusted means were calculated with analyses of covariance. We used linear regression analyses to assess whether components of homocysteine metabolism (homocysteine, *S*-adenosylmethionine, *S*-adenosylhomocysteine, the ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine, folate, vitamin B_6 and vitamin B_{12}) were associated with intima-media thickness. Since many associations between potential determinants and intima-media thickness were non-linear, we compared the intima-media thickness in tertiles of the determinants. In the regression analyses we compared the upper tertile with the lower tertiles. Firstly, we calculated crude associations with intima-media thickness as outcome variable and components of homocysteine metabolism as respective determinants (Model 1). Secondly, we adjusted these associations for potential confounders. Therefore, we added age, sex, systolic blood pressure, creatinine

clearance, total cholesterol and waist-to-hip ratio as possible determinants, the selection of which was based on literature data (Model 2) [20]. In additional analyses we adjusted associations for other cardiovascular risk factors (HDL-cholesterol, triglycerides, insulin, smoking, body mass index and prior cardiovascular disease). Subsequently, we mutually adjusted the analyses with homocysteine, *S*-adenosylmethionine or *S*-adenosylhomocysteine, and performed additional adjustment for B vitamins. We stratified the analyses for the presence of type 2 diabetes to assess whether regression coefficients were different for diabetic and non-diabetic individuals. For reasons of statistical power the 22 individuals with an impaired glucose tolerance were regarded as non-diabetic individuals, because they did not materially differ from individuals with a normal glucose tolerance with regard to cardiovascular risk factors. If regression coefficients differed between diabetic and non-diabetic participants we tested for interaction by adding a product term to the regression analyses.

3. Results

Table 1 lists the demographic and clinical characteristics of non-diabetic and diabetic participants. Diabetic individuals were younger, had a higher creatinine clearance, systolic blood pressure and waist-to-hip ratio, and had a lower cholesterol concentration. Homocysteine concentration was slightly, but significantly lower in diabetic subjects than in non-diabetic subjects. Concentrations of folate, *S*-adenosylmethionine and *S*-adenosylhomocysteine in erythrocytes were significantly higher in diabetic subjects. Mean intima-media thickness did not differ significantly between diabetic and non-diabetic individuals and was similar to that in two previous studies [21,22]. Among the non-homocysteine-related variables, only age, systolic blood pressure and creatinine clearance showed correlations with intima-media thickness at the $P < 0.10$ level both in non-diabetic and diabetic individuals (data not shown in detail).

3.1. Components of homocysteine metabolism as determinants of intima-media thickness

Among diabetic individuals only, the highest tertile of plasma homocysteine was significantly associated with a greater intima-media thickness, which remained significant after adjustment for potential confounders (Table 2a). A homocysteine concentration above 12 μmol/l was associated with a 0.07 mm (95% CI, 0.01–0.13) greater intima-media thickness. Age and creatinine clearance were the major confounders.

Table 2b shows that the highest tertile of erythrocyte *S*-adenosylmethionine was associated with a smaller intima-media thickness, which was significant only among non-diabetic individuals. After adjustment for cardiovascular risk factors, this association remained significant. An *S*-adenosylmethionine concentration above 4000 nmol/l was associated with a smaller intima-media thickness of 0.12 mm (95% CI, 0.20–0.036) in non-diabetic individuals. Age and systolic blood pressure were major confounders, whereas creatinine clearance was a modest confounder. *S*-adenosylmethionine in plasma and *S*-adenosylhomocysteine in plasma and erythrocytes were not significantly associated with intima-media thickness in either diabetic or non-diabetic individuals (data not shown).

Intima-media thickness was not associated with the ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine in erythrocytes and plasma, nor with vitamin B_6, vitamin B_{12} or folate in plasma or erythrocytes (data not shown).

Fig. 1 depicts the mean intima-media thickness per tertile increase in plasma homocysteine concentration and *S*-adenosylmethionine concentration in erythrocytes, for diabetic and non-diabetic individuals separately.

3.2. Additional analyses

Regression analyses were repeated with additional adjustment for HDL-cholesterol, triglycerides, insulin, smoking, body mass index and prior cardiovascular disease. These additional analyses did not materially alter the results (data not shown). Additional adjustment of the association between homocysteine, *S*-adenosylmethionine or *S*-adenosylhomocysteine and intima-media thickness for other components of homocysteine metabolism (including B vitamins) also did not alter the associations reported in Table 2 (data not shown). Finally, we repeated the analyses with exclusion of participants with known diabetes. This did not change the results (data not shown).

4. Discussion

The salient findings of this study are (1) that we found a significant positive association between plasma homocysteine and intima-media thickness only among diabetic individuals, which was independent of classical cardiovascular risk factors and of other components of homocysteine metabolism; and (2) that we observed an inverse association between *S*-adenosylmethionine in erythrocytes and intima-media thickness, which was statistically significant only among non-diabetic individuals and, again, independent of classical cardiovascular risk factors and of other components of homocysteine metabolism. Taken together, these findings may provide an explanation, at least in part, for the increased

Table 1
Characteristics of the participants

	Mean (S.D.[a]), median (IQR[b]) or percentage		P-value	Age-adjusted P-value
	Non-diabetic individuals $n = 91$	Diabetic individuals $n = 140$		
Age (years)	71 (6)	67 (7)	< 0.001	
Gender (% male)	56	50	0.44	0.12
Systolic blood pressure (mmHg)	143 (18)	148 (20)	0.07	0.003
Creatinine clearance (ml/min)	67 (13)	80 (20)	< 0.001	0.002
Body mass index (kg/m^2)	26.2 (3.5)	28.7 (3.8)	< 0.001	< 0.001
Waist-to-hip ratio (men)	0.98 (0.06)	1.00 (0.06)	0.03	0.006
Waist-to-hip ratio (women)	0.85 (0.07)	0.91 (0.08)	< 0.001	< 0.001
Smoking (%)	20.2	14.8	0.30	0.28
Plasma or serum value				
Total cholesterol (mmol/l)	5.8 (0.9)	5.5 (1.1)	0.04	0.01
HDL-cholesterol (mmol/l)	1.4 (0.4)	1.2 (0.3)	< 0.001	< 0.001
Triglycerides (mmol/l)	1.3 (1.0–1.7)	1.7 (1.2–2.2)	< 0.001	< 0.001
Insulin (pmol/l)	55.0 (40.5–73.5)	87.0 (61.0–118.0)	< 0.001	< 0.001
Homocysteine (μmol/l)	11.0 (9.6–13.8)	10.2 (8.4–12.5)	0.01	0.16
Vitamin B_6 (nmol/l)	36 (24–51)	34 (24–46)	0.58	0.59
Vitamin B_{12} (pmol/l)	267 (213–313)	278 (221–342)	0.27	0.63
Folate (nmol/l)	14.5 (11.1–18.9)	15.9 (12.4–20.8)	0.07	0.24
S-adenosylmethionine (nmol/l)	97 (82–107)	94 (82–107)	0.79	0.33
S-adenosylhomocysteine (nmol/l)	15.2 (12.5–17.8)	15.3 (12.2–18.3)	0.93	0.17
SAM/SAH[c] ratio	6.3 (5.5–7.0)	6.1 (5.1–7.2)	0.57	0.13
Erythrocyte value				
Folate (nmol/l)	528 (394–632)	564 (443–688)	0.04	0.18
S-adenosylmethionine (nmol/l)	3495 (3040–3923)	3786 (3437–4300)	< 0.001	0.002
S-adenosylhomocysteine (nmol/l)	132 (111–160)	144 (119–178)	0.023	0.06
SAM/SAH[c] ratio	25.5 (21.3–32.2)	27.1 (19.7–32.8)	1.00	0.79
Intima-media thickness	0.88 (0.2)	0.86 (0.2)	0.41	0.28

Reference ranges: vitamin B_6, 17–100 nmol/l; vitamin B_{12}, 156–672 pmol/l; folate in erythrocytes, 125–2500 nmol/l; for *S*-adenosylmethionine and *S*-adenosylhomocysteine in plasma and erythrocytes no generally accepted reference ranges exist.

[a] Standard deviation.

[b] Interquartile range.

[c] Ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine.

vascular susceptibility of diabetic individuals to hyperhomocysteinemia.

A positive association between a high homocysteine concentration and intima-media thickness was found among diabetic individuals only. This association was not explained by other determinants of intima-media thickness (such as age and blood pressure), nor by *S*-adenosylmethionine, *S*-adenosylhomocysteine, folate, vitamin B_6 or vitamin B_{12} or renal function. Previous studies of homocysteine and intima-media thickness in non-diabetic populations have [21,23–26] or have not [27,28] found a significant association. The association in the ARIC Study [23] was not significant after adjustment for other cardiovascular risk factors. In addition, Bots et al. adjusted only for age and sex, which might have overestimated the association [21]. Taken together, these studies show that, among non-diabetic individuals, homocysteine is not consistently associated with intima-media thickness, and that other variables may confound or modify such an association. Only two reports have focused on homocysteine and intima-media thickness in type 2 diabetes, and found that plasma homocysteine concentration and a genetic mutation in the enzyme methylenetetrahydrofolate reductase (MTHFR) were not significantly associated with carotid intima-media thickness [29,30]. However, the associations between homocysteine and intima-media thickness were positive in these studies, and lack of statistical significance may have been due to chance.

To our knowledge, there are no reports on whether *S*-adenosylmethionine, *S*-adenosylhomocysteine or their ratio are determinants of intima-media thickness. We found that a high concentration of *S*-adenosylmethionine in erythrocytes was associated with a lower intima-media thickness, but only among non-diabetic individuals. A plausible explanation for this finding may be that a high concentration of *S*-adenosylmethionine represents more methyl group substrate availability for methylation reactions, which are crucial to many biochemical processes, including repair of damaged proteins [5]. Although there is no detailed molecular explanation of how cellular hypomethylation could contribute to atherosclerosis, the biochemical complexity of the atherosclerotic process and the various cell

Table 2
Multiple linear regression analyses

	Non-diabetic individuals		Diabetic individuals	
	Beta	95% CI	Beta	95% CI
(*a*) *Homocysteine* (*highest*[a] *vs. lower two tertiles*) *as determinant of intima-media thickness* (*in mm*)				
Model 1	0.038	−0.039 to 0.12	0.095[b]	0.022–0.15
Model 2	−0.0039	−0.076 to 0.068	0.070[c]	0.01–0.13
(*b*) *S-adenosylmethionine in erythrocytes* (*highest*[d] *vs. lower two tertiles*) *as determinant of intima-media thickness*				
Model 1	−0.14[e]	−0.23 to −0.048	−0.057	−0.12 to 0.0020
Model 2	−0.12[f]	−0.20 to −0.036	−0.041	−0.099 to 0.017

P for interaction (diabetes *upper vs. lower tertiles of homocysteine) = 0.28. Variables entered: Model 1: homocysteine; Model 2: as model 1 plus age, gender, systolic blood pressure, creatinine clearance, waist-to-hip ratio and total cholesterol. *P* for interaction (diabetes *upper vs. lower tertiles of *S*-adenosylmethionine in erythrocytes) = 0.11. Variables entered: Model 1: *S*-adenosylmethionine in erythrocytes; Model 2: as model 1 plus age, gender, systolic blood pressure, creatinine clearance, waist-to-hip ratio and total cholesterol.

[a] > 12 μmol/l.
[b] *P*-value = 0.004.
[c] *P*-value = 0.028.
[d] > 4000 nmol/l.
[e] *P*-value = 0.003.
[f] *P*-value = 0.005.

types involved by no means preclude a role for hypomethylation. We did not find a 'protective' effect of *S*-adenosylmethionine in diabetic individuals, suggesting that it may be overruled by cardiovascular risk factors associated with the diabetic state per se. In contrast to *S*-adenosylmethionine in erythrocytes, we did not find an association between intima-media thickness and *S*-adenosylhomocysteine or the ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine in erythrocytes. Loehrer et al. reported a positive association between a low ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine in erythrocytes and peripheral arterial disease [10]. A low ratio may be a better reflection of methylation status than *S*-adenosylmethionine alone, because it contains information on both available methyl groups (conferred by the concentration of *S*-adenosylmethionine), as well as on substrate inhibition of transmethylation reactions (conferred by the concentration of *S*-adenosylhomocysteine). The absence of any degree of correlation between high *S*-adenosylhomocysteine levels and intima-media thickness caused the *S*-adenosylmethionine to *S*-adenosylhomocysteine ratio to be a poor predictor of intima-media thickness in our study. Taken together, these previous [10] and present data suggest that cellular hypomethylation is associated with atherosclerosis. However, whether this is best reflected by *S*-adenosylmethionine levels or by the ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine requires further study.

Our results are in line with the observations that homocysteine is a risk factor for cardiovascular disease particularly in individuals with type 2 diabetes [2–4]. These previous studies, one of which was prospective [4], as well as our observations regarding intima-media thickness, indicate that individuals with diabetes may be particularly susceptible to the detrimental effects of homocysteine on the vascular wall, i.e. the atherosclerotic component of atherothrombosis. The molecular explanations for this increased susceptibility remain to be defined. Firstly, it may include an acceleration of glucose-induced oxidative stress on endothelial cells by hyperhomocysteinemia. Secondly, it may include impaired cellular methylation affecting biosynthesis of a wide range of endogenous compounds, such as proteins, DNA and RNA [10]. Support for the first hypothesis was recently provided by Shukla et al., who demon-

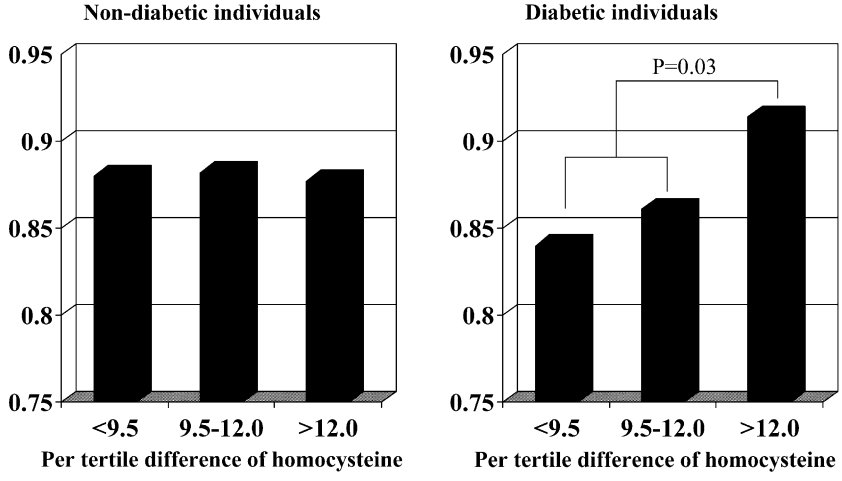

Fig. 1. Mean value of intima-media thickness (in mm) per tertile difference of homocysteine in plasma and *S*-adenosylmethionine in erythrocytes after adjustment for age, gender, systolic blood pressure, creatinine clearance, waist-to-hip ratio and total cholesterol. Significant *P*-values for the difference in mean value between the highest and lower two tertiles are displayed.

strated that concentrations of homocysteine that had no effect on aortae of normal rabbits markedly impaired endothelium-dependent relaxation and nitric-oxide–(NO–) dependent cyclic GMP formation (an index of endothelial NO bioactivity) in aortae of diabetic rabbits. This effect was reversible by addition of superoxide dismutase [31]. Another animal study showed that 10 μmol/l homocysteine was sufficient to inhibit NO formation in diabetic rat aortae, whereas in aortae of nondiabetic animals concentrations as high as 1 mmol/l were required to elicit similar effects [32]. The potentiation of diabetic angiopathy by homocysteine could, therefore, be mediated by impairment of NO formation, possibly by the overproduction of superoxide. Support for the second hypothesis was provided by Poirier et al., who observed a linear correlation between erythrocyte concentrations of *S*-adenosylmethionine and lymphocyte MTHFR activity among individuals without diabetes [33]. The latter enzyme catalyzes the last step in the formation of methyl groups from the folate pool, which are necessary for synthesis of methionine (and thus for *S*-adenosylmethionine). Among participants with diabetes (also among those without complications), this correlation was absent, suggesting an altered *S*-adenosylmethionine regulation among individuals with diabetes. In addition, among participants with diabetes, progression of nephropathy was associated with a lower MTHFR activity in lymphocytes. Unfortunately, this study was quite small and analyses in this study may have been biased because no correction was made for age, whereas age clearly differed between the groups under investigation [33]. In summary, these two hypotheses may (partly) explain why individuals with diabetes are particularly susceptible to the detrimental effects of homocysteine on carotid IMT. For future research, it would be interesting to study *S*-adenosylmethionine, *S*-adenosylhomocysteine, homocysteine and MTHFR activity in endothelial cells, because impairment of endothelial function is a key event in current models of atherothrombosis. In addition, such a study should include individuals both with and without diabetes.

We did not observe an association between concentrations of folate (in erythrocytes or plasma), vitamin B_6 or vitamin B_{12} and intima-media thickness. This is in line with findings of two earlier reports on folate and intima-media thickness [24,29].

This study had some limitations. Firstly, we only measured fasting homocysteine concentrations. Thus, we do not know whether the findings also apply to non-fasting or post-methionine homocysteine, *S*-adenosylmethionine and *S*-adenosylhomocysteine concentrations. Secondly, the power of this study was probably too small to demonstrate an interaction between type 2 diabetes and homocysteine or *S*-adenosylmethionine.

Taken together, our study identifies two components of the increased vascular susceptibility of patients with type 2 diabetes to alterations in homocysteine metabolism. The first component is the increased vulnerability to homocysteine itself, the second is the lack of a protective effect of high *S*-adenosylmethionine concentrations. Further studies are needed to define the molecular mechanisms that underlie these findings.

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